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Citation for published version:

McGorum, B, Scholes, S, Milne, E, Eaton, S, Wishart, T, Poxton, I, Moss, S, Wernery, U, Davey, T, Harris, J & Pirie, S 2016, 'Equine grass sickness, but not botulism, causes autonomic and enteric neurodegeneration and increases SNARE protein expression within neuronal perikarya', *Equine Veterinary Journal*, vol. 48, no. 6, pp. 786-791. <https://doi.org/10.1111/evj.12543>

Digital Object Identifier (DOI):

[10.1111/evj.12543](https://doi.org/10.1111/evj.12543)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Equine Veterinary Journal

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Equine grass sickness, but not botulism, causes autonomic and enteric neurodegeneration and increases SNARE protein expression within neuronal perikarya

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Authors' declaration of interests

No competing interests have been declared.

Source of funding

This project was funded by the RCVS Trust Blue Sky Research Fund, The Equine Grass Sickness Fund and The Dubai Millennium Research Foundation.

Acknowledgements

The authors thank Neil McIntyre for performing immunohistochemistry.

Word count; 3731

Authorship – All authors qualify for authorship on the grounds of the following; (i) study design [BM, SS, EM, IP, UW, TD, JH, SP], (ii) study execution [BM, SS, EM, IP, SM, UW, TD, JH, SP], (iii) data analysis and interpretation [BM, TD, EM, SS, JH], (iv) preparation of the manuscript [BM, SS, JH], and (v) final approval of the manuscript [all authors]. TW and SE performed QFWB analysis and interpretation.

Summary

Reasons for performing study: Equine grass sickness (EGS) is of unknown aetiology. Despite some evidence suggesting that it represents a toxico-infection with *Clostridium botulinum* types C and/or D, the effect of EGS on the functional targets of botulinum neurotoxins (BoNTs), namely the soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins, is unknown. Further, while it is commonly stated that, unlike EGS, equine botulism is not associated with autonomic and enteric neurodegeneration, this has not been definitively assessed.

Objectives: To determine (a) whether botulism causes autonomic and enteric neurodegeneration, and (b) the effect of EGS on the expression of SNARE proteins within cranial cervical ganglion [CCG] and enteric neuronal perikarya.

Methods: Light microscopy was used to compare the morphology of neurons in haematoxylin-eosin stained sections of CCG and ileum from 6 EGS horses, 5 botulism horses and 6 control horses. Immunohistochemistry was used to compare the expression of synaptosomal-associated protein-25 (SNAP-25), synaptobrevin (Syb) and syntaxin (Syn) within CCG neurons, and of Syb in enteric neurons, from horses with EGS, horses with botulism and control horses. The concentrations of these SNARE proteins in extracts of CCG from EGS and control horses were compared using quantitative fluorescent western blotting (QFWB).

Results: EGS, but not botulism, was associated with autonomic and enteric neurodegeneration and with increased immunoreactivity for SNARE proteins within neuronal perikarya. QFWB confirmed increased concentrations of SNAP-25, Syb and Syn within CCG extracts from EGS versus control horses, with the increases in the latter two proteins being statistically significant.

Conclusions and potential relevance: The occurrence of autonomic and enteric neurodegeneration, and increased expression of SNARE proteins within neuronal perikarya, in EGS but not botulism, suggests that EGS may not be caused by BoNTs. Further investigation of the aetiology of EGS is therefore warranted.

Keywords: horse; grass sickness; botulism; SNARE proteins; SNAP-25; syntaxin; synaptobrevin.

Introduction

Equine grass sickness (EGS) is widely hypothesised to be a toxico-infectious form of botulism, whereby a dietary trigger induces intestinal overgrowth of *Clostridium botulinum* C and/or D, with resultant *in vivo* production of botulinum neurotoxins (BoNTs) [1-4]. Consistent with this hypothesis, EGS is associated with an increased frequency of detection of *C. botulinum* type C and BoNT/C1 within the intestinal tract [2]. Furthermore, increased serum levels of antibodies specific for botulinum surface antigens and for BoNT/C1 have been detected in horses with recent exposure to EGS [3], and have been associated with increased protection against EGS [4]. A botulinum vaccine appeared to provide protection against EGS [1]. It has also been hypothesised that botulinum C2 toxin, an ADP-ribosylating toxin produced by *C. botulinum* C and D, may contribute to neurodegeneration in EGS [2,3].

In all species, BoNTs cause generalised neuromuscular dysfunction by cleaving the soluble N-ethylmaleimide sensitive fusion attachment receptor (SNARE) proteins which are essential for presynaptic vesicle exocytosis, leading to accumulation of synaptic vesicles in nerve terminal boutons and the consequent failure of synaptic transmission [5]. BoNTs are remarkably specific proteases, with BoNT/B, /D, /F and /G cleaving the vesicle protein synaptobrevin

(Syb), BoNT/A and /E cleaving the plasma membrane protein SNAP-25 and BoNT/C cleaving both synaptosomal-associated protein-25 (SNAP-25) and the peripheral plasma membrane protein syntaxin (Syn) [6].

The authors question the proposed role of BoNTs in EGS, given the obvious differences in clinical signs between EGS and neuroparalytic botulism [7]. Furthermore, it is anecdotally stated that the autonomic and enteric neurodegeneration which characterises EGS does not occur in equine botulism [7,8], although has not been definitively assessed. The aim of this study was to investigate further the potential role of BoNTs in EGS by using (a) conventional histological examination of CCG and ileal neurons to determine whether botulism causes autonomic and enteric neurodegeneration resembling that which characterises EGS, (b) immunohistochemistry to compare the expression of SNARE proteins in cranial cervical ganglion [CCG] neurons [SNAP-25, Syn, Syb] and enteric neurons [Syb], in EGS, botulism and control horses, and (c) quantitative fluorescent western blotting [QFWB] to compare the concentration of SNARE proteins in extracts of CCG from EGS and control horses.

Materials and methods

Samples

The study was approved by both the University of Edinburgh Ethical Review Committee and an ethical review committee within the United Arab Emirates (Permit 550J53). EGS and control horse samples were collected with owners' consent from horses that were subjected to euthanasia on humane grounds. CCG and ileum samples were collected from 6 EGS (4 acute EGS and 2 subacute EGS; median age 7 years, range 2-20) and 6 control (11, 6-15 years) mixed-breed and mixed-gender horses (Supplementary file 1). EGS was categorised as

previously described and confirmed by necropsy including histopathological examination of cranial cervical ganglia [9]. Control horses were euthanased for reasons other than neurological disease. Samples were also obtained from 5 horses (median age 11 years, range 6-19 years) with neuromuscular botulism. One of these horses had a clinical history, clinical signs, and (negative) post-mortem findings consistent with botulism, although toxin testing was not performed. Archived formalin fixed tissue blocks of CCG and ileums were also obtained from a previous historic study conducted in Dubai, from 4 horses which developed neuromuscular botulism following oral administration of a bacteria-free supernatant from a broth culture of *C. botulinum* type C (C1 and C2 producer). All samples were collected within 3 h of euthanasia. Samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax for histology and immunohistochemistry. CCG samples for QFWS were rapidly frozen by immersion on dry ice pellets and stored at -80°C pending analysis.

Light microscopy

Light microscopy of haematoxylin-eosin stained sections of all samples was used to compare morphology of CCG and enteric neurons in the 3 groups of horses and determine whether the neurons from botulism horses had the features of neurodegeneration which characterise EGS [10].

Immunohistochemical localisation of SNARE proteins in CCG and enteric neurons from horses with EGS, horses with botulism and control horses

CCG sections were labelled for SNAP-25, Syn and Syb, while ileum sections were labelled only for Syb, largely as previously described [11]. Tissues were dewaxed and rehydrated. Antigen retrieval was performed by heating sections in 250 ml citrate buffer (pH 6.0, 0.01 M) for 15 min in a pressure cooker, before cooling for 5 min in running water. A commercial

immunolabelling kit^a was used according to manufacturer's instructions. Slides were rinsed with Tris buffered saline containing 0.5 M Tween pH 7.5 (TBST) and incubated with primary antibodies overnight at 4°C. Primary antibodies were titrated to identify the lowest dilution that consistently yielded specific labelling in control horse sections. Primary antibodies were murine monoclonal anti-SNAP25^b diluted 1:1500 in diluent^c, murine monoclonal anti-Syn STX01HPC-1^q diluted 1:1600, and murine monoclonal anti-Syb^e diluted 1:1600. For negative control sections, antibody diluent^c replaced primary antibody. Slides were rinsed and incubated with peroxidase blocking agent^f for 10 min, horseradish peroxidase-labelled polymer for 40 min, then substrate-chromogen solution^g for 10 min with rinsing between each step. Slides were rinsed once in distilled water, counterstained with Harris's haematoxylin (1 min), dipped in Scott's tap water substitute, dehydrated, cleared using ethanol then xylene and mounted under DPX.

QFWB comparison of concentrations of SNARE proteins in CCG extracts from EGS and control horses

Unfortunately samples from botulism horses were unavailable for this part of the study. QFWB was done as previously described [12,13]. Briefly, protein was extracted in RIPA buffer^h containing 0.1% protease inhibitor cocktailⁱ from EGS (n=6) and control (n=6) horse CCG and the protein concentration determined using the Micro BCA Assay^j. CCG proteins (20µg load) were separated by SDS-polyacrylamide gel electrophoresis on 4–12% precast NuPage BisTris gradient gels^k and then transferred to PVDF membrane using an iBlot 2 fast transfer device^k. The membranes were then blocked using Odyssey blocking buffer^l and incubated with primary antibodies according to manufacturers' instructions (Table 2). Secondary antibodies (goat anti-rabbit IRDye 680 and donkey anti-mouse IRDye 680^l) were added according to manufacturers' instructions. Blots were imaged using an Odyssey Infrared

Imaging System¹. Scan resolution of the instrument ranges from 21 to 339 μm , and blots were imaged at 169 μm . Quantification was performed on single channels with the analysis software provided. Total protein stained gels were loaded in parallel with those used for membrane transfer, to ensure equal sample loading, and were analysed using the Odyssey Infrared Imaging system¹ as previously described [12].

Statistical analysis

Inter-group comparisons of age and QFWB data used, respectively, the Kruskal-Wallis and unpaired t-tests, with $P < 0.05$ indicating statistical significance.

Results

There was no significant inter-group difference in age.

Light microscopy

CCG and enteric neurons from EGS horses, but not control and botulism horses, had characteristic features of neuronal degeneration, including central to complete chromatolysis (loss of Nissl substance), neuronal swelling and vacuolation, intracytoplasmic eosinophilic inclusions, and eccentric and pyknotic nuclei (Fig 1).

Immunohistochemistry for SNARE proteins in CCG and enteric neurons

The distribution and density of labelling for all three SNARE proteins in CCG neurons was indistinguishable between control and botulism horses (Fig 2). In these horses, many neurons were unlabelled while others had faint punctate labelling confined to axons and to clusters of granules within soma. CCG neurons from EGS horses had variable intensity and distribution

of labelling, but there was increased intensity of labelling for all three proteins compared with botulism and control horses. The increased labelling of EGS neurons was most marked for Syb. EGS neurons had increased labelling throughout the soma or predominantly in the peripheral perikaryon, and frequently extending into proximal axons (Fig 2). Occasional EGS neurons had faint labelling that was indistinguishable from that of botulism and control horses. There was no apparent inter-group difference in the intensity of labelling of axons within CCG.

Submucosal and myenteric neuronal perikarya from control and botulism horses had fine granular labelling of Syb (Fig 3) and Syb immunopositive axons were noted in the muscularis, submucosa and coursing through villi. In EGS, there was increased intensity of Syb labelling in perikarya of degenerating submucosal and myenteric neurons, and positively labelled axons were noted in muscularis and submucosa but were virtually absent from villi.

QFWB analysis

QFWB analysis revealed significantly increased concentrations of Syb (EGS 170533 ± 46910 ; control 9973 ± 3123 , $P=0.0066$), Syn (EGS 49575 ± 4172 ; control 20775 ± 2076 , $P=0.0008$) and non-significantly increased concentrations of SNAP-25 (EGS 132344 ± 54101 ; control 68751 ± 27102 , $P\text{-value}=0.3238$) in CCG extracts from EGS horses compared with controls (Fig 4).

Discussion

This study confirmed anecdotal reports [7,8] that CCG and ileal enteric neurones are not chromatolytic in horses with botulism, in contrast to EGS, suggesting that EGS is unlikely to be caused by the particular BoNTs which caused neuroparalysis in the neuroparalytic botulism

cases. Since 4 of 5 botulism cases were associated with BoNT/C1 intoxication, these data are inconsistent with the hypothesis that EGS is a toxico-infection associated with BoNT/C1 producing *C. botulinum*, unless this toxin has different mechanisms of action when produced *in vivo* during a toxico-infection rather than following oral toxin exposure. Consistent with these findings, while experimental challenge with BoNT/C or BoNT/E, *in vitro* or *in vivo*, can cause neurodegeneration in other species [14-18], the threshold concentration for cytotoxicity is estimated to far exceed the lethal dose for humans and animals [19] and there is currently no evidence that natural (ie non-experimental) exposure to BoNTs can induce the neurodegeneration which characterises EGS.

Further evidence against a causal role for orally acquired BoNTs in EGS is the observation that EGS, but not botulism, was associated with increased neuronal expression of three target proteins for BoNTs, namely Syn, SNAP-25 and Syb. Perikarya of neurons from control horses and horses with botulism had faint punctate SNARE protein labelling distributed evenly throughout the cytoplasm, or confined to discrete clusters of granules which were often located in the juxtannuclear region. The latter may reflect labelling of SNARE proteins within the Golgi apparatus, since the morphology of the granule clusters resembles that of wheat germ agglutinin-labelled Golgi membranes [20]. In contrast, most EGS neurons had increased intensity of labelling of the entire soma, particularly peripherally. EGS horses also had loss of Syb immunoreactive nerve fibres within ileal mucosa, consistent with the previously reported profound loss of mucosal PGP 9.5 immunoreactive fibres in this region [21].

Consistent with increased immunoreactivity for SNARE proteins in CCG neuronal perikarya in EGS, QFWB analysis revealed increased abundance of all 3 SNARE proteins in extracts of CCG from EGS compared with those from control horses, with inter-group differences in Syb and Syn concentrations being statistically significant. These findings are consistent with previous proteomic data which showed increased abundance of SNAP-25 (2.155 fold

increase) in extracts of EGS versus control CCG; Syn and Syb were not identified in the proteome in this study [22]. Unfortunately extracts of CCG from horses with botulism were unavailable for QFWB.

The increased expression of SNARE proteins in EGS neuronal perikarya mirrors that of synaptophysin, which also accumulates within perikarya of CCG and enteric neurons from EGS horses, but not in those with botulism [11]. Synaptophysin is an abundant integral membrane protein of synaptic vesicles, while Syn, SNAP-25 and Syb are synapse-specific proteins predominantly localised to plasma membranes of axon terminals [23]. These presynaptic proteins are synthesized in neuronal perikarya, glycosylated in the Golgi apparatus and transported in a vesicular form to nerve terminals by axonal transport involving the cytoskeleton [24]. We hypothesise that the accumulation of synaptophysin and the three SNARE proteins in perikarya of neurons in EGS reflects (a) dysfunction of glycoprotein processing in the Golgi, (b) failure of axonal transport of protein-containing vesicles to nerve terminals, and/or (c) upregulation of neuronal synthesis of these proteins. Consistent with the first two hypotheses, ultrastructural loss of a recognizable Golgi structure is a likely early event in EGS, and EGS is associated with major perturbations in the cytoskeleton of autonomic neurons resulting in accumulation of dopamine- β -hydroxylase in neuronal perikarya [25].

The increased expression of SNARE proteins in neuronal perikarya in EGS, and not in botulism, is inconsistent with the hypothesis that BoNT/C causes EGS. Indeed, immunoreactivity of particular *intact* SNARE proteins at neuromuscular junctions is reduced following proteolytic cleavage by particular BoNTs [26,27]. The authors are unaware of reports that these BoNTs cause accumulation of SNARE proteins within neuronal perikarya. Furthermore, as BoNT/C1 does not alter Golgi ultrastructure or inhibit vesicle trafficking

within soma [28], it would not be expected to increase the concentration of these proteins in CCG neuronal perikarya.

While the findings of this study are inconsistent with EGS being caused by BoNT/C1, the data do not preclude involvement of other botulinum toxins. While the non-neurotoxic C2 and C3 adenosine diphosphate (ADP) ribosylating toxins have been proposed as a cause of cytoskeletal disruption and neurodegeneration in EGS [2], their involvement in EGS has not been assessed. While this study included archived tissues from 4 horses with botulism that had received oral culture supernatant from a C1 and C2 producing strain of *C. botulinum*, conclusions regarding the potential involvement of C2 in EGS cannot be made because it is not known whether the supernatant contained C2. Furthermore, C2 affects a wide range of cell types [29] and thus appears unlikely to selectively target the specific populations of neurons undergoing degeneration in EGS. The role of C3 in the pathogenesis of (non-experimental) diseases, including EGS, is unclear because it lacks an obvious cell-entry mechanism [30]. Further investigation of the aetiology of EGS is therefore warranted.

Manufacturers' addresses

^aDakoCytomation EnVision+ System-HRP; DAB K4001, Dako, Ely, UK.

^bab53723, AbCam, Cambridge, UK.

^cS0809, Dako, Ely, UK.

^dab3265, AbCam, Cambridge, UK.

^eab11104, AbCam, Cambridge, UK.

278 ^fReal Peroxidase blocker S2023, Dako, Ely, UK.

279 ^gImmPact Dab SK4105, Vector Laboratories, Peterborough, UK.

280 ^hThermo Scientific, Paisley, UK.

281 ⁱProtease inhibitor cocktail, Sigma, Gillingham, UK.

282 ^jMicro BCA Assay, Pierce, Thermo Scientific, Paisley, UK.

283 ^kNuPage BisTris gradient gels, Invitrogen, Thermo Scientific, Paisley, UK.

284 ^lLI-Cor Biosciences, Cambridge, UK.

285

286 **Figure legends**

287 **Figure 1:** Light micrograph of CCG (a-c) and enteric myenteric plexus (d-f) neurons from
288 EGS, botulism and control horses. Neurons from EGS horses (c, f), but not control (a,d) and
289 botulism (b, e) horses, were frequently degenerate involving chromatolysis (loss of Nissl
290 substance), neuronal swelling and vacuolation, intracytoplasmic eosinophilic inclusions, and
291 eccentric and pyknotic nuclei. Haematoxylin-eosin. Bars = 25µm.

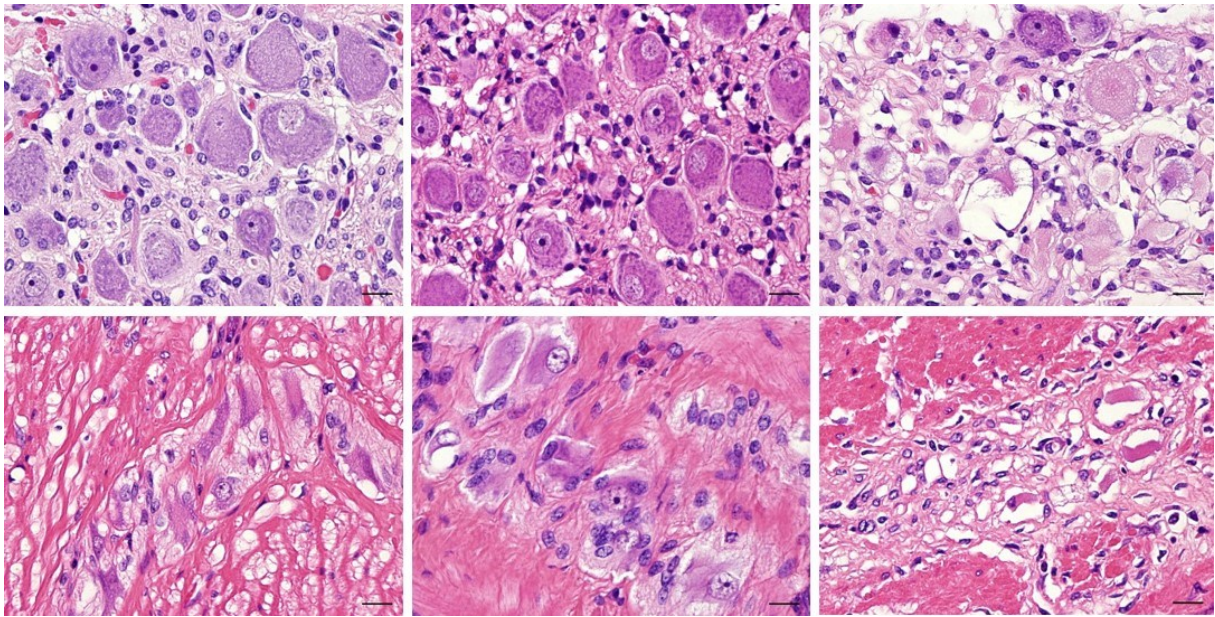


Figure 2: Immunohistochemical localisation of SNAP (a-c), Syb (d-f) and Syn (g-i) in CCG from control (a, d, g), botulism (b, e, h) and EGS (c, f, i) horses. Neurons from botulism and control horses were unlabelled or had faint punctate labelling confined to axons and to clusters of granules within soma. Neurons from EGS horses had variable intensity and distribution of labelling, but the intensity of labelling for all three proteins was increased compared with that for botulism and control horses.

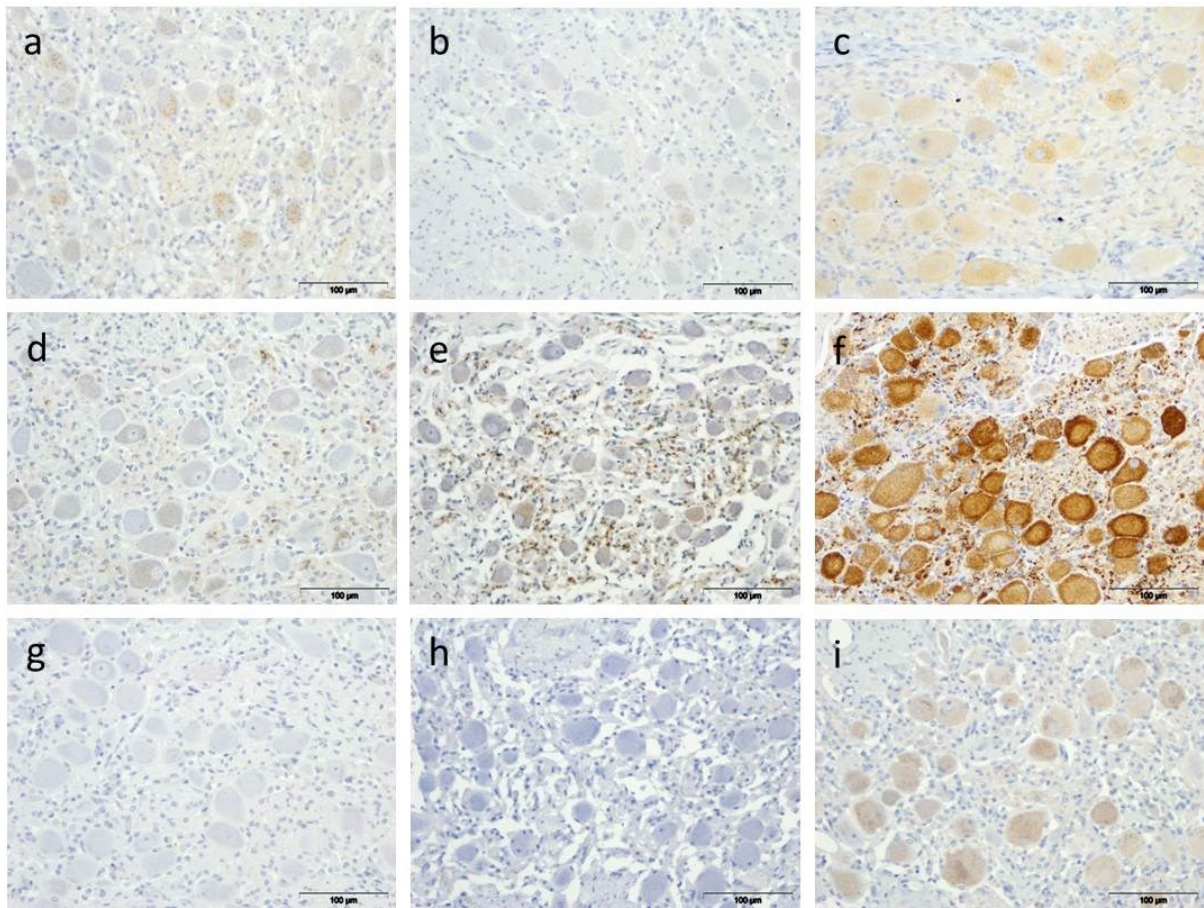


Figure 3: Immunohistochemical localisation of Syb in submucosal plexus neurons from (a) control, (b) botulism and (c) EGS horses. Syb immunoreactivity is confined mainly to axons in botulism and control horses, while neuronal perikarya from EGS horses have increased Syb immunoreactivity. Neuronal perikarya are labelled with short solid arrows, while long broken arrows label axons.

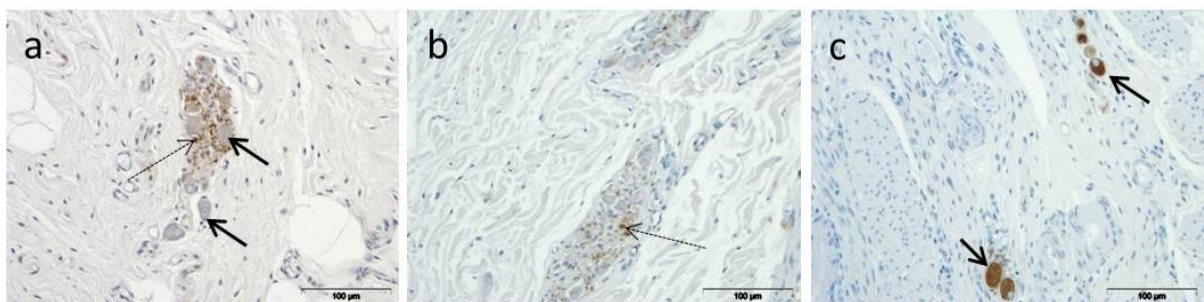
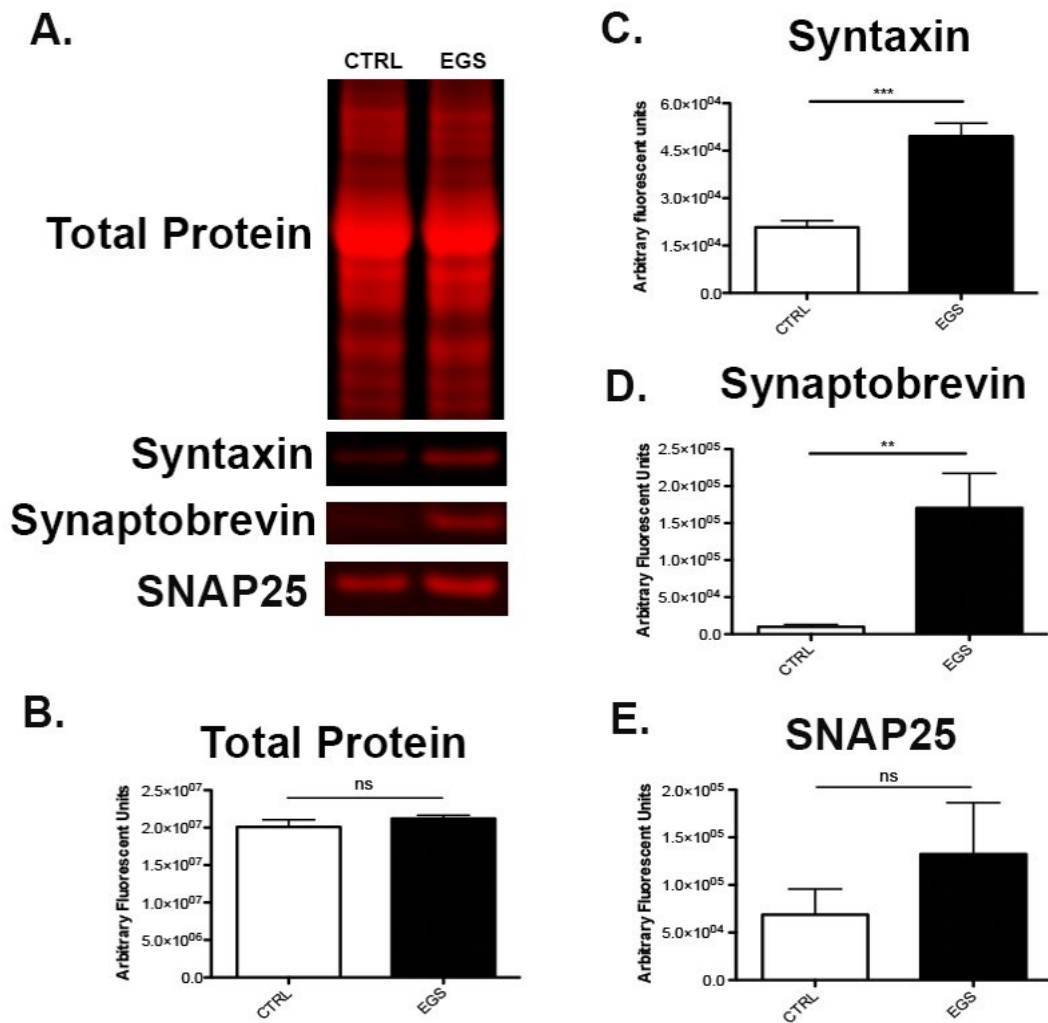


Figure 4: QFWB confirmed increased expression of synaptic proteins Syn (Panel A&C), Syb (Panel A&D) and SNAP-25 (Panel A&E) in CCG of EGS horses (n=6) relative to controls (CTRL, n=6). Total protein stained gel (A - top panel) demonstrates uniform sample loading and is confirmed by quantification (Panel B). ** P=0.0066, *** P=0.0008, NS=non-significant.



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